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# ORIGINAL ARTICLE

# Vaccination with *Klebsiella pneumoniae*-derived extracellular vesicles protects against bacteria-induced lethality via both humoral and cellular immunity

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The emergence of multidrug-resistant *Klebsiella pneumoniae* highlights the need to develop preventive measures to ameliorate *Klebsiella* infections. Bacteria-derived extracellular vesicles (EVs) are spherical nanometer-sized proteolipids enriched with outer membrane proteins. Gram-negative bacteria-derived EVs have gained interest for use as nonliving complex vaccines. In the present study, we evaluated whether *K. pneumoniae*-derived EVs confer protection against bacteria-induced lethality. *K. pneumoniae*-derived EVs isolated from *in vitro* bacterial culture supernatants induced innate immunity, including the upregulation of co-stimulatory molecule expression and proinflammatory mediator production. EV vaccination via the intraperitoneal route elicited EV-reactive antibodies and interferon-gamma-producing T-cell responses. Three vaccinations with the EVs prevented bacteria-induced lethality. As verified by sera and splenocytes adoptive transfer, the protective effect of EV vaccination was dependent on both humoral and cellular immunity. Taken together, these findings suggest that *K. pneumoniae*-derived EVs are a novel vaccine candidate against *K. pneumoniae* infections.
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## INTRODUCTION

*Klebsiella pneumoniae* is a Gram-negative, encapsulated, facultative anaerobic bacterium, which is found in the normal flora of the mouth, skin and intestine.[1] The most common infection caused by the *Klebsiella* bacterium outside of hospitals is pneumonia, typically in the form of bronchopneumonia and bronchitis.[2] *K. pneumoniae* can cause destructive changes to the human lungs via inflammation and hemorrhage, which can produce a thick, bloody, mucoid sputum.[3] This bacterium gains access to the lower respiratory tract typically after a person aspirates microbes that colonize the oropharyngeal mucosa. *Klebsiella* infections are observed mostly in people with a weakened immune system and cause a high death rate of ~ 50%, even with antimicrobial therapy.[4,5] In addition to pneumonia, *K. pneumoniae* can also cause infections in the urinary tract, lower biliary tract and surgical wound sites.[6–8] In recent years, *K. pneumoniae* has become an important pathogen in nosocomial infections. Intense antibiotic use is a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria, which are often resistant to multiple antibiotics.[9,10]

Fatal infections caused by multidrug-resistant *K. pneumoniae* have created a pressing need to develop a preventive measure to ameliorate *Klebsiella* infections.

Vaccination is the administration of antigenic material to produce protective immunity to a disease. Vaccination is regarded as the most cost-effective method for preventing infectious diseases. This approach requires the induction of protective immunity, which is best achieved using an active immunization strategy with the ability to induce specific long-term protective memory, the hallmark of adaptive immunity. The key to eradicate pathogenic bacterial infections is the activation of specific immune responses, such as humoral (or antibody-mediated) and cellular (or T-cell-mediated) immunity. Gram-negative bacteria-derived extracellular vesicles (EVs), otherwise known as outer membrane vesicles, are spherical bilayered phospholipids 20–200 nm in diameter that harbor many proteins related to pathogenesis.[11–13] Biochemical and proteomic studies have revealed that bacterial EVs are composed of outer membrane proteins, lipopolysaccharide, outer membrane lipids, periplasmic proteins, DNA, RNA and

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other factors associated with virulence.[14–16] Recent studies have revealed the potential of EVs derived from Gram-negative bacteria, such as *Neisseria meningitidis*, *Acinetobacter baumannii*, *Porphyromonas gingivalis*, *Salmonella enterica* serovar Typhimurium, *Helicobacter pylori* and *Vibrio cholerae*, in inducing protective immunity against bacterial infections in mice.[17–25]

*K. pneumoniae* secretes outer membrane vesicles that can induce the innate immune response.[26] Various outer membrane proteins can be used as the candidate antigens for vaccine.[27–29] In the present study, we evaluated whether *K. pneumoniae*-derived EVs elicit a protective effect against *Klebsiella* infections. This study showed that vaccination with *K. pneumoniae*-derived EVs effectively protected bacteria-induced lethality in a murine sepsis model. In addition, this study clarified the underlying mechanisms of how EV vaccination provides protection against *Klebsiella*-induced sepsis.

## MATERIALS AND METHODS

### Mice and cell cultures

Six to seven-week-old female, wild-type C57BL/6 (genetic background from The Jackson Laboratory, Bar Harbor, ME, USA) were used in the present study. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Pohang University of Science and Technology, Pohang, Republic of Korea with approval number 2011-01-0017. A total of $2 \times 10^6$ Raw 264.7 mouse macrophages (purchased from ATCC, Manassas, VA, USA) were cultured with Dulbecco's Modified Eagle's medium (DMEM) (Hyclone, Little Chalfont, UK) containing 10% fetal bovine serum (FBS) and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) in 100 mm tissue culture dishes (TPP, Trasadingen, Swiss) at 37 °C until use in an experiment. Extracted bone marrow-derived dendritic cells were cultured with Roswell Park Memorial Institute (RPMI) 1640 (Hyclone) containing 10% FBS, 50 μM 2-mercaptoethanol (ME) and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin). For the *ex vivo* experiments, RPMI 1640 (Hyclone) containing 10% FBS, 50 μM 2-ME, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) was used to culture the splenocytes.

### Bacteria strain and preparation of *K. pneumoniae*-derived EVs

*K. pneumoniae* (ATCC 4208) was purchased from ATCC and was cultured in nutrient broth (Merck, Kenilworth, NJ, USA) at 37 °C shaking at 200 rpm to an optical density of 1.5. The cultured bacteria were pelleted twice at $10\,000 \times g$ for 20 min at 4 °C. The supernatants were filtered using a 0.45-μm bottle top vacuum filter (Corning, Corning, NY, USA) and were concentrated with a 100-kDa hollow fiber membrane filter using the QuixStand Benchtop System (Amersham Biosciences, Little Chalfont, UK). The concentrated solution was filtered using a 0.22-μm bottle top vacuum filter (Corning) to remove any remaining bacteria. The final filtered solution was ultracentrifuged at $150\,000 \times g$ for 3 h at 4 °C in a type 45 Ti rotor (Beckman Instruments, Brea, CA, USA). Finally, the EV pellet was re-suspended in phosphate-buffered saline (PBS) and was quantitated using the Bradford assay (BIO-RAD, Hercules, CA, USA). The prepared EVs were stored at − 80 °C until use.[30] All EV batch preparations were performed under the same conditions.

### Transmission electron microscopy

The EVs were diluted with PBS, and 10 μl of the suspension (50 μg ml$^{-1}$) were dropped on 300-mesh copper grids (EMS, Hatfield, PA, USA). Uranyl acetate (2%) was then dropped onto the grids to stain the EVs. The images were captured using a JEM1011 electron microscope (JEOL, Tokyo, Japan).[31]

### Dynamic light scattering

The EVs were diluted with PBS to 500 ng ml$^{-1}$, and the diameter size distribution was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) and Dynamic V6 software.[32]

### Macrophage uptake assay

The EVs were incubated with 2 μM of DiI (Invitrogen, Waltham, MA, USA) for 1 h and were washed with PBS. The washed EVs were ultracentrifuged using a TLA 120.2 (Beckman Instruments) at $150\,000 \times g$ at 4 °C for 3 h. The pellet was re-suspended in PBS. Raw 264.7 mouse macrophages ($2 \times 10^5$ cells per well) were incubated with DMEM (Hyclone) containing 10% FBS and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) on a gelatin-coated cover class in a 24-well cell culture plate for 24 h and were then treated 10 μg ml$^{-1}$ of EVs for 1 and 6 h with DMEM containing antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin). After the stimulation, the cells were washed with PBS and fixed with 4% paraformaldehyde. For staining of the cell nucleus, the fixed cells were treated with 20 μM Hoechst (Sigma-Aldrich, St Louis, MO, USA) and were mounted on a glass slide. A LSM 510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany) was used to acquire the confocal images.[33]

### *In vitro* macrophage studies

Raw 264.7 mouse macrophages ($5 \times 10^5$ cells per well) were incubated with DMEM containing 10% FBS and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) in a 24-well tissue culture plate at 37 °C for 24 h. The culture medium was removed and various doses of EVs (0.1–1000 ng/ml$^{-1}$) in serum-free media (DMEM) containing antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) were added. After a 15 h stimulation, the culture medium was collected and the levels of proinflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor-alpha, were measured.

### Expression of cell surface molecules on bone marrow-derived dendritic cells

To obtain mouse bone marrow-derived dendritic cells, mouse femurs were extracted and bone marrow was washed with cold PBS using a sterile syringe on a non-coated petri dish (SPL, Pocheon, Korea). At day 0, the extracted bone marrow-derived dendritic cells ($2 \times 10^6$ cells per plate) were cultured with 10 ml of RPMI 1640 (Hyclone) containing 20 ng ml$^{-1}$ of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA), 10% FBS, 50 μM 2-ME and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin). At day 3, 10 ml of the same culture medium was added to the plate. At days 6 and 8, 10 ml of the culture medium were collected, and the cells were pelleted by centrifugation. Each pellet was re-suspended in 10 ml of same medium and was added to the plate. At day 10, the cells were harvested by centrifugation. At day 11, the cells were transferred to a 24-well tissue culture plate for stimulation. The prepared cells ($5 \times 10^5$ cells per well in 24-well plate) were

incubated with RPMI 1641 containing 10% FBS, 50 μM 2-ME and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin). After 24 h, the culture medium was removed, and then the various stimulators (lipopolysaccharide (Sigma-Aldrich) 10 100 ng ml$^{-1}$; EVs 0.1 pg ml$^{-1}$-1000 ng ml$^{-1}$) in serum-free medium (RPMI 1641 containing 2-ME and antibiotics) were added to the cells. After 24 h of stimulation, the cells were stained with anti-CD11c-APC (BD Biosciences, Franklin Lakes, NJ, USA), anti-CD86-PE (BD Biosciences), or anti- major histocompatibility complex class II-fluorescein isothiocyanate (BD Biosciences) antibodies. A FACSCalibur (BD Biosciences) instrument was used for fluorescence-activated cell sorting analysis.[13]

### Lethal dose determinations of *K. pneumoniae*-induced bacterial sepsis

For determining the lethal dose of *K. pneumonia* infections, three different doses ($1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$) of *K. pneumoniae* strain in 100 μl of PBS were injected intraperitoneally into mice. The survival rate was observed every 12 h for 3 days.

### Evaluation of the protective effect of vaccination

For confirming the immune response to EVs *in vivo*, the mice were injected intraperitoneally with 10, 100 and 1000 ng of *K. pneumoniae*-derived EVs in 100 μl of PBS three times for 3 weeks at 1-week intervals. Seven days after the last immunization, the spleens and blood were extracted for analysis using re-stimulation assay and to measure antibody titer, respectively. To confirm the protective effect of vaccination against bacterial infection, the mice were injected intra-peritoneally with 0.5, 1 and 2 μg of EVs in 100 μl of PBS for the same period of time. Seven days after the last immunization, both the immunized and non-immunized (sham) mice were challenged with the lethal dose of $1 \times 10^8$ colony forming units (CFU) of *K. pneumoniae*. The survival rate was observed every 12 h for 3 days.

### Splenocyte re-stimulation assay

The extracted spleens were mashed with the plunger of 5-ml syringe onto a 100 μm cell strainer (BD Biosciences) with washing buffer (DMEM containing 2.5% FBS and 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The separated spleen cells were treated with an ammonium chloride solution (STEM CELL, British Columbia, Canada) for 10 min at 4 °C to lyse the red blood cells. The splenocytes were washed with washing buffer and were then filtered through a 40 μm cell strainer (BD Biosciences). Finally, the cells were incubated with RPMI 1640 containing 10% FBS, 50 μM 2-ME, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and antibiotics (100 unit ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin) in a 24-well plate (TPP) coated with 1 μg ml ml$^{-1}$ of anti-CD3 (eBioscience, San Diego, CA, USA) and 1 μg ml$^{-1}$ of anti-CD28 (eBioscience) antibodies for 12 h to re-stimulate the splenic T cells.

### Measurement of the EV antibody titer

EVs (100 ng) in 100 μl PBS were coated in a black 96-well plate (Greiner Bio-one, Monroe, NC, USA) for 24 h at 4 °C. The EV-coated plate was washed with PBS and blocked with the reagent diluent (PBS containing 1% BSA; Roche) for 1 h. After washing with PBS, the extracted naive (sham) and vaccinated mouse blood samples were centrifuged to separate the serum and cell fractions. The serum was diluted with the reagent diluent (1:500) and was then incubated in the EV-coated plate for 2 h. After incubation, the plates were washed and treated with 200 ng ml$^{-1}$ of horseradish peroxidase-conjugated goat

anti-mouse immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology, CA, USA) for 2 h. After washing the plates, the enhanced chemiluminescence substrate (Thermo Scientific, Kenilworth, NJ, USA) was added and analyzed using a Victor Wallac 1420 apparatus (PerkinElmer, Waltham, MA, USA).

### Intracellular cytokine staining of splenic T cells

For the intracellular cytokine staining assay, the mice were immunized with three doses (10, 100 and 1000 ng) of *K. pneumoniae* EVs using the above protocol. A week after the last immunization, the spleens of both immunized and non-immunized mice were extracted for use in the re-stimulation assay. The separated splenocytes were incubated in 48-well plates coated with 1 μg ml$^{-1}$ of anti-CD3 (eBioscience) and anti-CD28 (eBioscience) antibodies at 37 °C for 12 h. The splenocytes were stained with anti-CD3-APC (BD Biosciences) and anti-CD4-FITC (BD Biosciences) antibodies for 30 min at 4 °C followed by fixation for 10 min in 4% paraformaldehyde at room temperature. The splenocytes were then incubated with antibodies against the cytokines of interest (anti-interferon (IFN)-γ-PE, anti-IL-17-PE and anti-IL-4-PE; BD Biosciences) for 30 min at room temperature followed by analysis using a FACSCalibur flow cytometer (BD Biosciences) with the CELLQuest software.[34]

### Adoptive transfer of sera and splenocytes

The mice were immunized intraperitoneally with 1 μg of EVs three times for 3 weeks. Seven days after the last immunization, blood and spleens were extracted. The same method was used to acquire the serum and splenocytes. The extracted serum (100 μl per mouse) and splenocytes ($1 \times 10^8$ cells per mouse) were injected intraperitoneally into naive mice. At 24 h after the injection, $2.5 \times 10^7$ and $5 \times 10^7$ CFU of the bacteria administered intraperitoneally were used to challenge the mice transferred with serum, splenocytes or sham (PBS). The survival rate was observed every 12 h for 3 days

### Measurement of cytokines

The cytokine levels secreted from Raw 264.7 cells or mouse-derived splenocytes were measured using a DuoSet ELISA according to the manufacturer's protocol (R&D Systems).

### Statistical analysis

The survival curves were compared using the log-rank test. All of the values are expressed as the mean ± s.e.m., and the Student's *t*-test was used to calculate *P*- values based on comparisons with the appropriate control.

## RESULTS

### Characterization of *K. pneumoniae*-derived EVs

To characterize EVs derived from *K. pneumoniae*, the EVs were extracted from the bacterial culture. Transmission electron microscopy analysis revealed that *K. pneumoniae* secreted EVs, which are spherical and contain a lipid-bilayer (Supplementary Figure 1a). Dynamic light scattering analysis revealed a mean diameter of 36 nm (Supplementary Figure 1b).

### *In vitro* innate immune response induced by *K. pneumoniae*-derived EVs

Host defense relies on the concerted action of both innate immunity and antigen-specific adaptive immunity. We first evaluated innate immune responses induced by *K. pneumoniae*
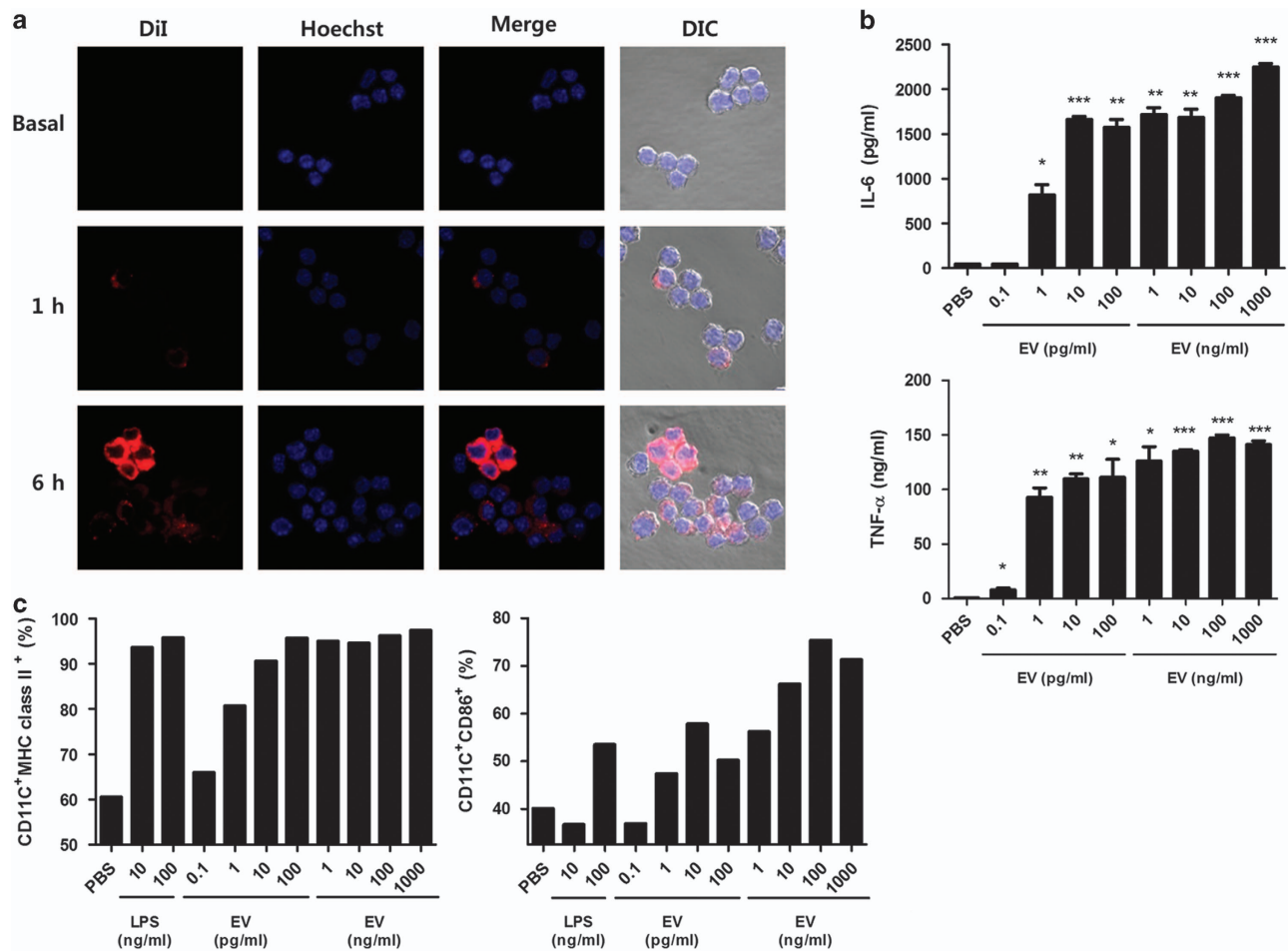
Figure 1 *In vitro* immunogenicity of *K. pneumoniae* EVs. (**a**) Raw 264.7 mouse macrophage uptake of EVs. Row 1 is the non-treated group. Row 2 and 3 are treated with EVs for 1 and 6 h, respectively. Column 1 shows DiI-stained EVs, column 2 shows the Hoechst-stained cell nucleus, column 3 shows the merged DiI and Hoechst images, and column 4 are the DIC-merged images. (**b**) Secreted IL-6 and TNF-α from Raw 264.7 cells stimulated by different doses of EVs measured with ELISA. (n = 2; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) (**c**) The expression of MHC class II and CD86 molecules on the surface of CD11C+ bone marrow-derived dendritic cells.

EVs. When macrophages were treated with EVs for 1 and 6 h, the signals against EVs were observed in the cytoplasm of macrophages, indicating that the EVs were internalized by antigen-presenting cells (Figure 1a). Moreover, the EV-treated macrophages efficiently induced the production of proinflammatory cytokines, such as IL-6 and tumor necrosis factor-alpha (Figure 1b). The treatment of macrophages with EVs induced an increase in the expression of co-stimulatory major histocompatibility complex class II and CD86 molecules (Figure 1c and Supplementary Figure 2). These results suggested that *K. pneumoniae* EVs effectively activate innate immune responses by antigen-presenting cells, which can prime T cells.

### *In vivo* antibody production induced by vaccination with *K. pneumoniae* EVs

To determine the effective dose of EVs for vaccination, we assessed the effect of EV immunization on the induction of antigen-specific antibody and T-cell responses. During the immunization process, sera were obtained from EV- and sham-immunized mice, and the EV-reactive IgG titer was also

evaluated (Figure 2a). An increase in *K. pneumoniae* EV-reactive IgG was detected after immunization with 10, 100 or 1000 ng of *K. pneumoniae* EVs. However, *K. pneumoniae* EV-reactive IgG was not detected in the sera from mice immunized with *Escherichia coli* EVs, whereas *E. coli* EV-reactive IgG increased in these mice (Figure 2b). These findings indicated that *K. pneumoniae* EV vaccination induced the production of IgG antibody specific for *K. pneumoniae* EVs.

### *In vivo* T-cell responses induced by *K. pneumoniae* EV vaccination

Next, to evaluate EV-specific T-cell responses, splenocytes were isolated from EV- and sham-immunized mice 1 week after the last immunization and were then re-stimulated with anti-CD3 and anti-CD28 antibodies for 12 h. The EV-specific production of IFN-γ, the key cytokine produced by Th1 cells, was enhanced in EV-immunized mice in a dose-dependent manner vs. the control (Figure 2c). However, EV-specific production of IL-17 and IL-4, the key cytokine of Th17 and Th2 cells, respectively, was not enhanced by vaccination (Figure 2c).
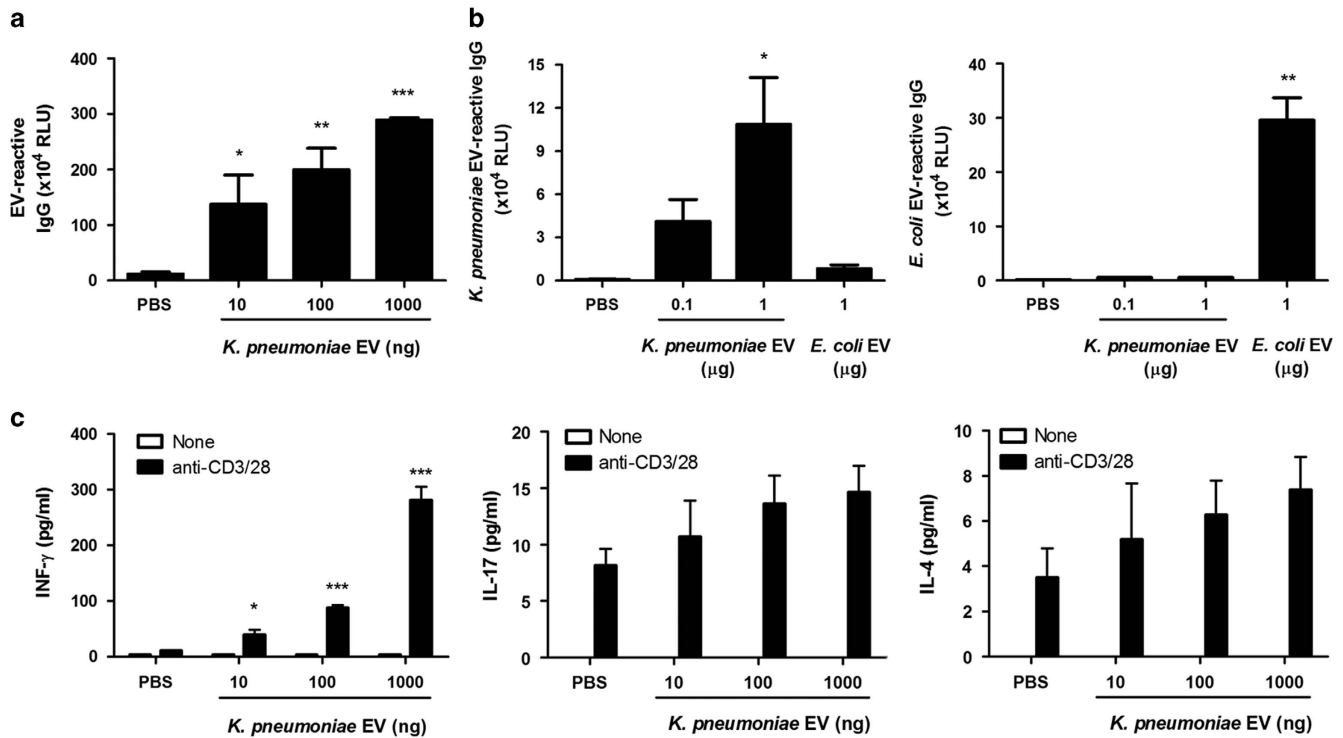
**Figure 2** *In vivo* immunogenicity of *K. pneumoniae* EVs. (**a**) *K. pneumoniae* EV-reactive IgG antibody in serum from mice immunized with 10, 100, 100 ng of *K. pneumoniae* EVs. This antibody was measured by ELISA ($n = 5$; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, compared with PBS group). (**b**) *E. coli* EV-reactive and *K. pneumoniae* EV-reactive IgG antibody in serum from mice immunized with 0.1 and 1 µg of *K. pneumoniae* EVs and 1 µg of *E. coli* EVs ($n = 5$; $*P < 0.05$ and $**P < 0.01$, compared with PBS group). (**c**) Secreted IFN-γ, IL-17 and IL-4 from splenocytes with and without stimulation with anti-CD3 and anti-CD28 antibodies. The mice were immunized with 10, 100, 1000 ng of *K. pneumoniae* EVs ($n = 5$; $*P < 0.05$ and $**P < 0.01$, compared with the non-stimulated, PBS-injected group).

We also evaluated EV-specific T-cell responses using intracellular cytokine staining of splenocytes 12 h after the *in vitro* stimulation with anti-CD3 and anti-CD28 antibodies, as shown in Supplementary Figure 3a. This study showed that the expression of IFN-γ, IL-17 and IL-4 in CD4+ cells was enhanced by *K. pneumoniae* EV immunization vs. sham immunization (Figures 3a and b). Together, these findings suggest that immunization with *K. pneumonia*-derived EVs induces a strong Th1, IFN-γ-producing T-cell response in addition to Th17 and Th2 responses against EV antigens.

## Effect of *K. pneumoniae*-derived EV vaccination on protection against bacteria-induced sepsis

To test the efficacy of EV vaccination against *K. pneumoniae*-induced sepsis, a mouse sepsis model was established by intraperitoneal injection with the different doses of *K. pneumoniae* (Figure 4a, left panel). The lethal dose of *K. pneumoniae* in this model was $1 \times 10^8$ CFU (Figure 4a, right panel). To verify the ability of *K. pneumoniae*-derived EVs as an effective vaccine candidate, we examined the effect of EV immunization on the development *K. pneumoniae*-induced sepsis (Figure 4b, left panel). Mice were injected intraperitoneally with different doses of the EV vaccine candidate every week for 3 weeks, were challenged with the lethal dose of *K. pneumoniae* ($1.0 \times 10^8$ CFU) 7 days after the final immunization, and the mice were then monitored every 12 h

for 5 days for survival. All of the mice immunized with sham died 36 h after the bacteria challenge; however, 80% of the mice survived after vaccination with 0.5 µg of the EVs and 100% of mice survived after vaccination with 1 µg of the EVs. An increase in the EV quantity (2 µg) did not improve the vaccine efficacy (Figure 4b). Therefore, the 1 µg dose was chosen for the vaccine dosage.

## Adoptive transfer of sera and splenocytes for protection against *K. pneumonia*-induced lethality

Adoptive transfer is one alternative mean to induce immunization. To determine whether antibody-mediated humoral or T-cell-mediated cellular immunity is important in conferring the EV-induced vaccination effect, we evaluated the protective effect of adoptive serum and splenocyte transfers as shown in Supplementary Figure 3b. Naive mice that received splenocytes from EV-immunized mice effectively protected the bacteria with a 100% and 40% survival rate after $2.5 \times 10^7$ and $5 \times 10^7$ CFU of *K. pneumoniae* challenge, respectively, whereas 40% and 0% of mice receiving splenocytes from sham-immunized mice survived the $2.5 \times 10^7$ and $5 \times 10^7$ CFU challenge, respectively (Figures 5a and b). In addition, the naive mice that received sera from EV-immunized mice had an enhanced ability to survive after *K. pneumoniae* challenge displaying 70% and 30% survival rate after $2.5 \times 10^7$ and $5 \times 10^7$ CFU of *K. pneumoniae* challenge, respectively, whereas 40% and 0% of
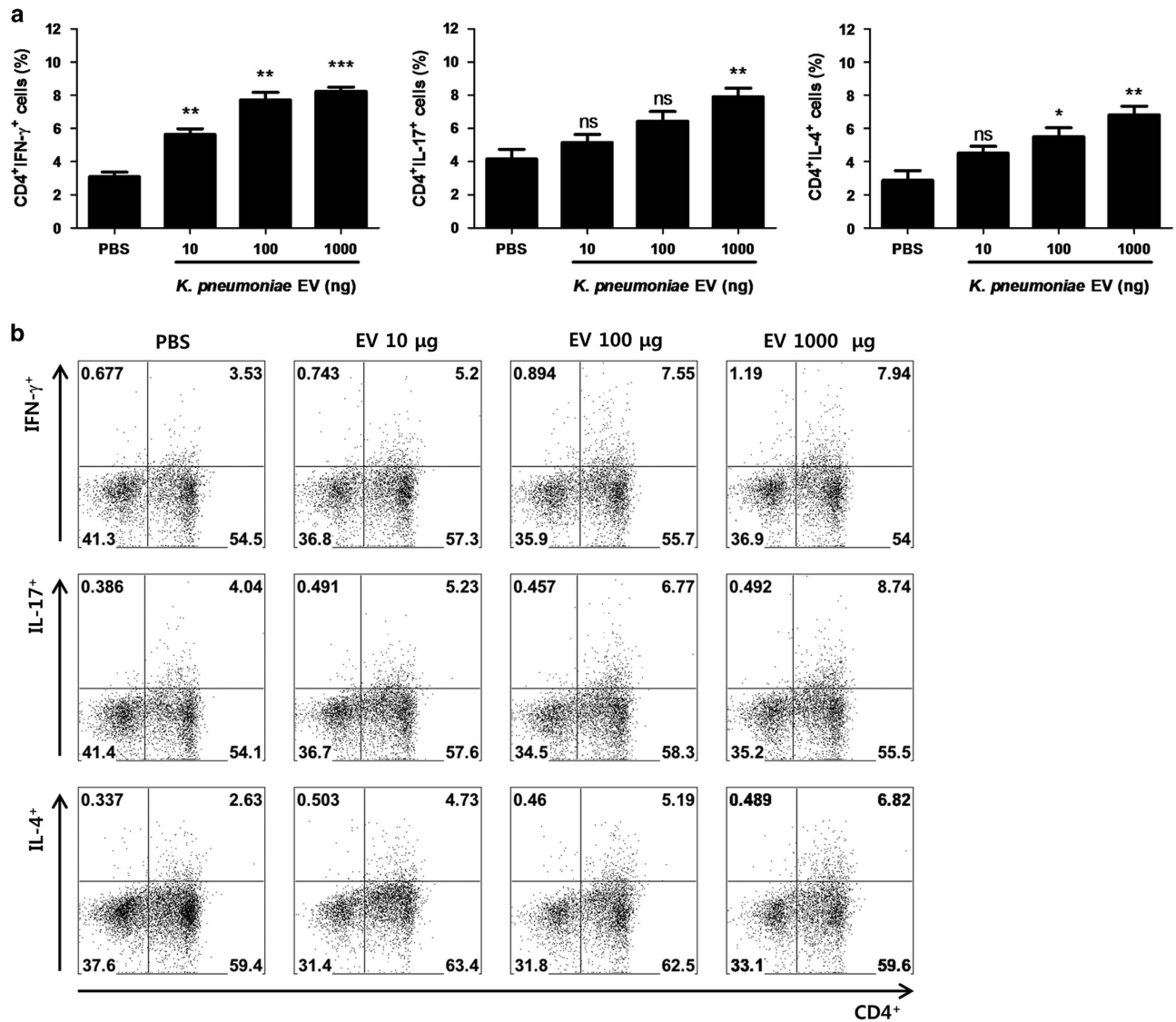
**a**



**b**



**Figure 3** Proportion of IFN-γ+, IL-17+ and IL-4+ CD4+ T cells. The mice splenocytes, which were immunized with 10, 100, 1000 ng of *K. pneumoniae* EVs three times for 3 weeks at a 1-week interval, were stimulated with anti-CD3 and anti-CD28 antibodies. (**a**) The graphs and (**b**) FACS dot plots of intracellular cytokine staining assay for IFN-γ, IL-17 and IL-4 in splenic CD4+ T cells. CD3+, CD4+ and cytokine-positive splenocytes were gated using APC, FITC and PE, respectively ($n=3$; *$P<0.05$, **$P<0.01$ and ***$P<0.001$, compared with PBS group).

mice receiving sera from the sham-immunized mice survived after the $2.5 \times 10^7$ and $5 \times 10^7$ CFU challenge, respectively (Figures 5a and b). These results suggest that both humoral and cellular immunity confer the protective effect of *K. pneumoniae* EV vaccination.

## DISCUSSION

Vaccination is considered to be the most effective and cost-effective method to prevent infectious diseases. In the present study, we aimed to evaluate the potential use of *K. pneumoniae* EVs as a new vaccine material to prevent lethality by *K. pneumoniae* infection. Vaccination with a non-lethal dose of *K. pneumoniae* EVs effectively protected against lethal infection with the same strain of *K. pneumoniae* via both humoral and cellular immunity. These protective effects were

accompanied by the involvement of innate and adaptive immune responses induced by EVs, which included EV-reactive IgG and IFN-γ-mediated immune responses. These findings suggest that native EVs derived from *K. pneumoniae* are a novel vaccine candidate for the prevention of *K. pneumoniae* infections.

*K. pneumoniae* is the most common cause of nosocomial respiratory tract and premature intensive care infections and the second most frequent cause of Gram-negative bacteremia.[35–37] Many studies have shown increasing resistance of this pathogen to antibiotics, resulting in an average rate of 1.63 outbreaks every year.[35] A variety of preventive measures have been attempted to reduce this incidence. However, passive immunization and immunoenhancers were shown to not be practical for the prevention *K. pneumoniae* infections.[38]
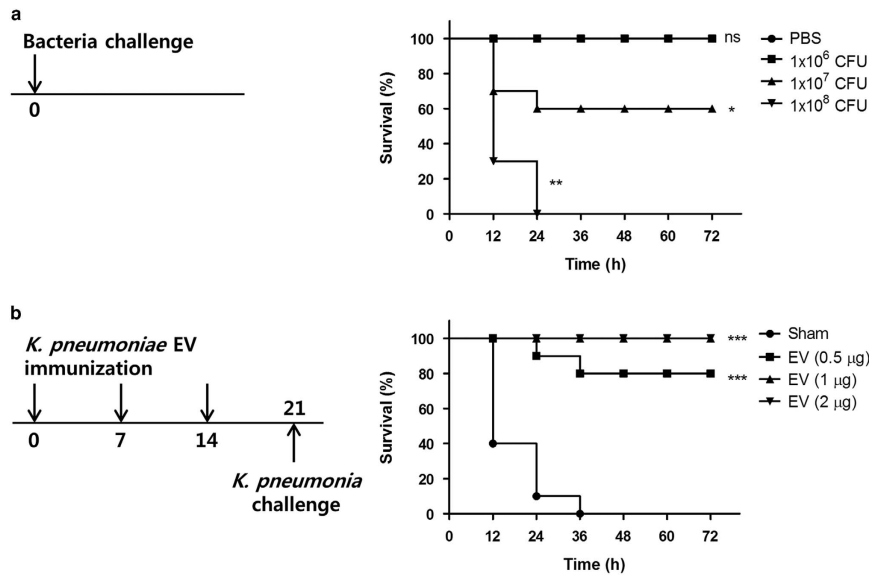
**Figure 4** Protective effect of *K. pneumoniae* EV immunization against *K. pneumoniae* infection. (**a**) Survival graph for determining the lethal dose of *K. pneumoniae*. Various doses ($1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$ and $1 \times 10^8$ CFU) of bacteria were injected intraperitoneally into mice ($n = 10$; $*P < 0.05$ and $**P < 0.01$, compared with the PBS-injected group). (**b**) Survival graph of $1 \times 10^8$ CFU of *K. pneumoniae* challenged mice after immunization with 0.5, 1 and 2 µg of *K. pneumoniae* EVs three times for 3 weeks at a 1-week interval ($n = 10$; $***P < 0.001$, compared with the sham mice group).



**Figure 5** Protective effect of adoptive transfer of sera and splenocytes from *K. pneumoniae* EV-immunized mice against lethal *K. pneumoniae* infection. Challenge with the bacteria was performed 24 h after the serum or splenocytes were transferred to the naive mice. Survival graph of serum or splenocyte transferred mice after challenged with $2.5 \times 10^7$ CFU of bacteria (**a**) and $5 \times 10^7$ CFU of bacteria (**b**) ($n = 10$, each group; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, compared with the sham group).

During the last 40 years, the construction of effective vaccines against *K. pneumoniae* has been attempted on numerous occasions. Among these efforts, capsular polysaccharide was targeted as a vaccine antigen, but the variable capsular polysaccharide serotypes limited their use in vaccine production.[39] The strategy of targeting non-capsular protein antigens revealed that outer membrane proteins are protective antigens in the immune response evoked by *K. pneumoniae*.[40] *K. pneumoniae* EVs may be an ideal vaccine candidate because they harbor many outer membrane proteins as well as immune stimulating pathogen-associated molecular patterns, including lipopolysaccharide. Indeed, the present study showed that *K. pneumoniae* EVs effectively protect against lethality induced by infection of the same strain of *K. pneumoniae*.

Adjuvant affects the quality and magnitude of immunological responses. Various adjuvants, including toll-like receptors 3, 4, 7 and 9 agonists, are used to enhance the immune response.[41] The presence of lipopolysaccharide in EVs emphasizes the ability of EVs to act as a natural adjuvant, thus allowing them to serve as potential adjuvant-free vaccine candidates for the delivery of various poorly immunogenic subunits.[42] The present study also showed that *K. pneumoniae* EVs enhance the expression of co-stimulatory molecules in macrophages and the production of proinflammatory mediators, which indicates that *K. pneumoniae* EVs have an adjuvant capacity in T-cell priming.

Antibody or T-cell responses have a key role in host defense toward *Klebsiella* infections.[43,44] Capsular polysaccharide from *K. pneumoniae* induces thymus-independent humoral immunity.[45] Animal experiments indicate that IFN-γ-deficient mice are more susceptible to bacterial infections compared with wild-type mice,[46,47] which suggests that the Th1-cell response is important in the protection against bacterial infections. Furthermore, IL-17-producing Th17 cells are necessary for vaccine-induced protection against bacterial

infections by enhancing neutrophil recruitment to infection sites.[48,49] These data suggest that protection against bacterial infections requires both phagocyte recruitment and phagocytosis mainly via Th17 cell and antibody-mediated immunity and also that the bactericidal activity of phagocytes is mediated mainly via Th1-cell immunity. Meanwhile, the present study showed that *K. pneumoniae* EV vaccination conferred protection against *K. pneumoniae* infection, which was dependent on both humoral and cellular immunity. Note that *K. pneumoniae* EV vaccination induces the production of IFN-γ more than that of IL-4 and IL-17 from T cells. These findings suggest that *K. pneumoniae* EV vaccination protects against bacterial infections mainly via both antibody and IFN-γ-mediated immune responses.

To the best of our knowledge, this is the first study to demonstrate *K. pneumoniae* EVs as a vaccine candidate against *K. pneumoniae* infections. Recently, we also found that *E. coli* EVs effectively protect against bacteria-induced sepsis.[13] Although the details of the mechanism underlying protection by EV vaccines still needs to be unraveled, the present study provides new insight for a novel strategy of vaccine development using Gram-negative bacteria-derived EVs to protect against bacterial infections, especially those caused by multi-drug-resistant bacteria.

## CONFLICT OF INTEREST
The authors declare no conflict of interest.

1 Guo Y, Cen Z, Zou Y, Fang X, Li T, Wang J *et al.* Whole-genome sequence of Klebsiella pneumonia strain LCT-KP214. *J Bacteriol* 2012; **194**: 3281.
2 White RJ, Blainey AD, Harrison KJ, Clarke SK. Causes of pneumonia presenting to a district general hospital. *Thorax* 1981; **36**: 566–570.
3 Kawai T. Hypermucoviscosity: an extremely sticky phenotype of Klebsiella pneumoniae associated with emerging destructive tissue abscess syndrome. *Clin Infect Dis* 2006; **42**: 1359–1361.
4 Borer A, Saidel-Odes L, Riesenberg K, Eskira S, Peled N, Nativ R *et al.* Attributable mortality rate for carbapenem-resistant Klebsiella pneumoniae bacteremia. *Infect Control Hosp Epidemiol* 2009; **30**: 972–976.
5 Gurntke S, Kohler C, Steinmetz I, Pfeifer Y, Eller C, Gastmeier P *et al.* Molecular epidemiology of extended-spectrum beta-lactamase (ESBL)-positive Klebsiella pneumoniae from bloodstream infections and risk factors for mortality. *J Infect Chemother* 2014; **20**: 817–819.
6 Ipekci T, Seyman D, Berk H, Celik O. Clinical and bacteriological efficacy of amikacin in the treatment of lower urinary tract infection caused by extended-spectrum beta-lactamase-producing Escherichia coli or Klebsiella pneumoniae. *J Infect Chemother* 2014; **20**: 762–767.
7 Ginawi I, Saleem M, Sigh M, Vaish AK, Ahmad I, Srivastava VK *et al.* Hospital acquired infections among patients admitted in the medical and surgical wards of a non-teaching secondary care hospital in northern India. *J Clin Diagn Res* 2014; **8**: 81–83.
8 Mpogoro FJ, Mshana SE, Mirambo MM, Kidenya BR, Gumodoka B, Imirzalioglu C. Incidence and predictors of surgical site infections following

caesarean sections at Bugando Medical Centre, Mwanza, Tanzania. *Antimicrob Resist Infect Control* 2014; **3**: 25.
9 Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 1998; **11**: 589–603.
10 Rodriguez EC, Saavedra SY, Leal AL, Alvarez C, Olarte N, Valderrama A *et al.* [The spread of KPC-3 Klebsiella pneumoniae in hospitals in Bogota over a three-year period (2008-2010)]. *Biomedica* 2014; **34**: 224–231.
11 Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev* 2005; **19**: 2645–2655.
12 Mashburn-Warren LM, Whiteley M. Special delivery: vesicle trafficking in prokaryotes. *Mol Microbiol* 2006; **61**: 839–846.
13 Kim OY, Hong BS, Park KS, Yoon YJ, Choi SJ, Lee WH *et al.* Immunization with Escherichia coli outer membrane vesicles protects bacteria-induced lethality via Th1 and Th17 cell responses. *J Immunol* 2013; **190**: 4092–4102.
14 Horstman AL, Kuehn MJ. Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway. *J Biol Chem* 2002; **277**: 32538–32545.
15 Lee EY, Choi DS, Kim KP, Gho YS. Proteomics in gram-negative bacterial outer membrane vesicles. *Mass Spectrom Rev* 2008; **27**: 535–555.
16 Wai SN, Lindmark B, Soderblom T, Takade A, Westermark M, Oscarsson J *et al.* Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell* 2003; **115**: 25–35.
17 Verkaik NJ, de Vogel CP, Boelens HA, Grumann D, Hoogenboezem T, Vink C *et al.* Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of Staphylococcus aureus. *J Infect Dis* 2009; **199**: 625–632.
18 Saunders NB, Shoemaker DR, Brandt BL, Moran EE, Larsen T, Zollinger WD. Immunogenicity of intranasally administered meningococcal native outer membrane vesicles in mice. *Infect Immun* 1999; **67**: 113–119.
19 Haneberg B, Dalseg R, Wedege E, Hoiby EA, Haugen IL, Oftung F *et al.* Intranasal administration of a meningococcal outer membrane vesicle vaccine induces persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans. *Infect Immun* 1998; **66**: 1334–1341.
20 Alaniz RC, Deatherage BL, Lara JC, Cookson BT. Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity *in vivo*. *J Immunol* 2007; **179**: 7692–7701.
21 McConnell MJ, Rumbo C, Bou G, Pachon J. Outer membrane vesicles as an acellular vaccine against *Acinetobacter baumannii*. *Vaccine* 2011; **29**: 5705–5710.
22 Dalseg R, Wedege E, Holst J, Haugen IL, Hoiby EA, Haneberg B. Outer membrane vesicles from group B meningococci are strongly immunogenic when given intranasally to mice. *Vaccine* 1999; **17**: 2336–2345.
23 Kesavalu L, Ebersole JL, Machen RL, Holt SC. Porphyromonas gingivalis virulence in mice: induction of immunity to bacterial components. *Infect Immun* 1992; **60**: 1455–1464.
24 Keenan J, Day T, Neal S, Cook B, Perez-Perez G, Allardyce R *et al.* A role for the bacterial outer membrane in the pathogenesis of Helicobacter pylori infection. *FEMS Microbiol Lett* 2000; **182**: 259–264.
25 Masignani V, Comanducci M, Giuliani MM, Bambini S, Adu-Bobie J, Arico B *et al.* Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870. *J Exp Med* 2003; **197**: 789–799.
26 Lee JC, Lee EJ, Lee JH, Jun SH, Choi CW, Kim SI *et al.* Klebsiella pneumoniae secretes outer membrane vesicles that induce the innate immune response. *FEMS Microbiol Lett* 2012; **331**: 17–24.
27 Kurupati P, Teh BK, Kumarasinghe G, Poh CL. Identification of vaccine candidate antigens of an ESBL producing Klebsiella pneumoniae clinical strain by immunoproteome analysis. *Proteomics* 2006; **6**: 836–844.
28 Lundberg U, Senn BM, Schuler W, Meinke A, Hanner M. Identification and characterization of antigens as vaccine candidates against Klebsiella pneumoniae. *Hum Vaccin Immunother* 2013; **9**: 497–505.
29 Hoppe S, Bier FF, von Nickisch-Rosenegk M. Identification of antigenic proteins of the nosocomial pathogen *Klebsiella pneumoniae*. *PLoS One* 2014; **9**: e110703.
30 Lee EY, Bang JY, Park GW, Choi DS, Kang JS, Kim HJ *et al.* Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli. *Proteomics* 2007; **7**: 3143–3153.
31 Park KS, Choi KH, Kim YS, Hong BS, Kim OY, Kim JH *et al.* Outer membrane vesicles derived from Escherichia coli induce systemic inflammatory response syndrome. *PLoS One* 2010; **5**: e11334.

32 Hallett FR, Watton J, Krygsman P. Vesicle sizing: number distributions by dynamic light scattering. *Biophys J* 1991; **59**: 357–362.

33 Ellis TN, Leiman SA, Kuehn MJ. Naturally produced outer membrane vesicles from Pseudomonas aeruginosa elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect Immun* 2010; **78**: 3822–3831.

34 Kim YS, Choi EJ, Lee WH, Choi SJ, Roh TY, Park J *et al*. Extracellular vesicles, especially derived from Gram-negative bacteria, in indoor dust induce neutrophilic pulmonary inflammation associated with both Th1 and Th17 cell responses. *Clin Exp Allergy* 2013; **43**: 443–454.

35 Ahmad TA, El-Sayed LH, Haroun M, Hussein AA, El Ashry el SH. Development of immunization trials against *Klebsiella pneumoniae*. *Vaccine* 2012; **30**: 2411–2420.

36 Lin WH, Tseng CC, Wu AB, Yang DC, Cheng SW, Wang MC *et al*. Clinical and microbiological characteristics of peritoneal dialysis-related peritonitis caused by *Klebsiella pneumoniae* in southern Taiwan. *J Microbiol Immunol Infect* 2013; **48**: 276–283.

37 Hawser S, Hoban DJ, Badal RE, Bouchillon SK, Biedenbach D, Hackel M *et al*. Epidemiology and antimicrobial susceptibility of Gram-negative aerobic bacteria causing intra-abdominal infections during 2010-2011. *J Chemother* 2014; **27**: 67–73.

38 Sun WS, Syu WJ, Ho WL, Lin CN, Tsai SF, Wang SH. SitA contributes to the virulence of *Klebsiella pneumoniae* in a mouse infection model. *Microbes Infect* 2014; **16**: 161–170.

39 Campbell WN, Hendrix E, Cryz S Jr, Cross AS. Immunogenicity of a 24-valent Klebsiella capsular polysaccharide vaccine and an eight-valent Pseudomonas O-polysaccharide conjugate vaccine administered to victims of acute trauma. *Clin Infect Dis* 1996; **23**: 179–181.

40 Bednarz-Misa I, Serek P, Dudek B, Pawlak A, Bugla-Ploskonska G, Gamian A. Application of zwitterionic detergent to the solubilization of *Klebsiella pneumoniae* outer membrane proteins for two-dimensional gel electrophoresis. *J Microbiol Methods* 2014; **107**: 74–79.

41 Fransen F, Boog CJ, van Putten JP, van der Ley P. Agonists of Toll-like receptors 3, 4, 7, and 9 are candidates for use as adjuvants in an outer membrane vaccine against Neisseria meningitidis serogroup B. *Infect Immun* 2007; **75**: 5939–5946.

42 Chen DJ, Osterrieder N, Metzger SM, Buckles E, Doody AM, DeLisa MP *et al*. Delivery of foreign antigens by engineered outer membrane vesicle vaccines. *Proc Natl Acad Sci USA* 2010; **107**: 3099–3104.

43 Kuenen JD, van Dijke EE, Hol C, Bootsma HJ, Verhoef J, van Dijk H. Protective effects of orally administered, Klebsiella-containing bacterial lysates in mice. *FEMS Immunol Med Microbiol* 1994; **8**: 69–75.

44 Montgomery CP, Daniels M, Zhao F, Alegre ML, Chong AS, Daum RS. Protective immunity against recurrent Staphylococcus aureus skin infection requires antibody and interleukin-17A. *Infect Immun* 2014; **82**: 2125–2134.

45 Alcantar-Curiel MD, Martinez-Ramos A, Garcia-Latorre E. [Capsular polysaccharide of Klebsiella pneumoniae. II. Immunogenic properties]. *Rev Latinoam Microbiol* 1993; **35**: 109–115.

46 Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon-gamma is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunology* 2001; **103**: 511–518.

47 Roberts LM, Davies JS, Sempowski GD, Frelinger JA. IFN-gamma, but not IL-17A, is required for survival during secondary pulmonary Francisella tularensis Live Vaccine Stain infection. *Vaccine* 2014; **32**: 3595–3603.

48 Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A *et al*. Relative contribution of Th1 and Th17 cells in adaptive immunity to Bordetella pertussis: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog* 2013; **9**: e1003264.

49 Gopal R, Rangel-Moreno J, Slight S, Lin Y, Nawar HF, Fallert Junecko BA *et al*. Interleukin-17-dependent CXCL13 mediates mucosal vaccine-induced immunity against tuberculosis. *Mucosal Immunol* 2013; **6**: 972–984.